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(57) Abstract

The present invention provides a novel sequence of the pig H-FABP gene, as well as methods of using said gene and its products. Especially the invention provides methods for detecting different alleles of the pig H-FABP gene, which different alleles are associated with differences in the genotypic and/or phenotypic traits of the pigs having those alleles. Especially the invention provides methods for distinguishing between alleles resulting in different phenotypes, particularly using techniques involving selective amplification of pig H-FABP gene derived materials. These techniques are especially suitable for selecting animals to be used in breeding programmes. Breeding programmes employing such techniques are also disclosed.

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THE PORCINE HEART FATTY ACID-BINDING PROTEIN ENCODING GENE AND METHODS TO IDENTIFY POLYMORPHISMS ASSOCIATED WITH BODY WEIGHT

INTRODUCTION

The invention relates to the field of molecular biology as well as to the field of breeding methods for farm animals, in particular pigs. In particular the invention relates to the use of diagnostic methods derived from the field of molecular biology to be applied in breeding programmes that select animals on production traits that improve their breeding value.

By selecting animals on their breeding value calculated mainly from phenotypic measurements of production traits, breeding has greatly improved the genotype for production traits of livestock animals. Thus, traditionally, breeding programmes have selected for phenotypic characteristics of animals. However, more recently selection for genotypic characteristics that are associated with improved production traits have gained interest in the field. Selection for phenotypic characteristics entails mainly selection of the offspring or siblings or other relatives of the animals to be selected whereas selection of specific genotypic characteristics allows for earlier and specific detection of animals of interest.

Within methods that select on specific genotypic characteristics, one may distinguish between methods that detect genetic variation in genes or quantitative trait loci that are merely associated with production traits of animals and methods that detect genetic variation in functional genes that directly influence those production traits. One of the former methods is a marker assisted selection wherein polymorphisms in markers identified in a random manner are associated with production traits.

For instance, meat production is closely linked to embryonic muscle formation, and, consecutively, to the distribution of muscle cells and fat cells. Biologically, production is concentrated in defined tissues of the animal, e.g. muscle tissue for lean meat production. In breeding programmes for optimizing porcine lean meat production, various levels of selection pressure have been applied to different tissues (i.e. muscle, fat and bone). However, when selecting for lean meat, and thus the abscence of fat, one may lose certain traits that are wanted after all, i.e. traits that are associated with taste and thus with the consumers perception of the final product.

In pig breeding programs traditionally a lot of emphasis has been put on the fat reduction because of the consumers interest in lean meat. Fat reduction is surveyed as a decrease in backfat thickness and a large reduction has been achieved since the establisment of breeding programmes in pigs. However reduction of the backfat depot also results in less intramuscular fat (IMF). This last depot is the main fat depot in meat and is positively correlated with the taste and thus the acceptance of meat (Wood et al., 1988). To exclude the IMF depot from further reduction a marker for this trait is necessary because IMF is hardly measurable in living animals. Recently, it has been statistically shown that a single major gene for IMF deposition in pigs must be present (Janss et al., 1994), however, the sequence, location and mode of action of the putative gene were not disclosed. Here we present evidence of a muscle tissue specific candidate gene located on porcine chromosome 6 which is the heart fatty acid-binding protein (H-FABP) gene. Genetic variation in this gene is responsible for the variation in among others IMF% and other production traits of pigs.

Fatty acid binding proteins (FABP's) are small intracellular proteins involved in fatty acid transport from the membrane to the sites of β oxidation and/or triacylglycerol or phospholipid synthesis (Veerkamp and

Maatman, 1995). Furthermore, FABP's modulated the intracellular fatty acid concentration (Veerkamp et al.,1993). Fatty acid metabolism has historically been linked to insuline resistance (Randle, 1963), and therefore mutations in FABP genes may be associated with changes in cellular insulin resistance or dependency, fatty acid oxydation and fatty acid binding. FABP's are members of a family of intracellular lipid binding proteins comprising at least eight structurally distinct types originating from: adipocytes, brain, epidermal cells, heart, intestinal cells, ileal cells, liver and myelin cells.

The heart type FABP (H-FABP) is a 15 kDa protein highly expressed in several tissues with a high demand for fatty acids, such as cardiac and skeletal muscle and lactating mammary gland. H-FABP is identical to MDGI (mammary derived growth inhibitor) a protein which inhibits growth of tumor cells *in vitro* (Bohmer et al., 1987). Functionally, the H-FABP can induce cardiac myocyte hypertrophy *in vitro*, when added to the culture (Burton et al.,1994) and also promotes functional differentiation of mammary epithelial cells *in vitro* (Yang et al.,1994). However, no secretion of H-FABP has been detected so far. On the other hand native and overexpression of H-FABP in mammary epithelium of lactating mice (*in vivo*) does not correlate with functional differentiation markers of these cells (Binas et al.,1995).

The present invention provides among others an isolated or recombinant pig H-FABP gene specific nucleic acid molucule or pig H-FABP gene specific fragments thereof comprising or hybridising to the nucleotide sequence as shown in figure 1, or its complementary sequence or the RNA equivalents thereof.

The locus of this gene is on porcine chromosome 6. The pig H-FABP gene can be assigned functions in the regulation of intramuscular fat, thereby changing the ratio of fat deposited within the muscle versus fat deposited outside the muscles, i.e. in backfat depots. Since production and deposition of fat is energy consuming and takes away energy

for other purposes, such as muscle growth, the regulation of intramuscular fat is correlated to the regulation of growth, and thus body weight and average daily gain and feed efficiency. Also, H-FABP can regulate myocyte (and thus muscle) hypertrophy and thus also muscle regeneration. Since FABP's are involved in fatty acid transport they can influence fatty acid oxidation rates, the metabolism of fatty acid derivatives in the tissue and the fatty acid composition of cells and thus of meat. Furthermore, FABP's may regulate cellular insulin dependency. Also, in pregnant animals, fat storage has an impact on embryo survival, and regulation of H-FABP will influence birth rates and littersize. Since H-FABP regulates functional differentiation of mammary epithelial cells it may be involved in regulating the quantity and composition of the milk available, thus influencing the growth and survival of newborn animals. With the present invention, the genetic variation within the pig H-FABP gene with respect to variation in regulation of expression can now be revealed and analysed for association with above production traits and physiological characteristics.

The present invention further provides a method to generate via recombinant DNA techniques an animal, such as small laboratory animals or farm animals, i.e. a pig, with additional genetic material originating from the pig H-FABP gene. Such animals may than encode wanted alleles of this gene and constitutively or transiently express allelic proteins or fragments thereof that enhance the production or physiological characteristics of those animals.

The invention further provides methods to generate proteins or (poly)peptides comprising various allelic proteins or fragments thereof derived from the pig H-FABP gene. Such peptides, or antibodies specifically directed against such peptides, may be used to influence production traits in the live animal, but may also be used in cell-culture systems *in vitro*. Such (poly)peptides or proteins, or antibodies specifically directed against these, may also

be used in diagnostic test systems to select animals that express wanted forms of allelic proteins or fragments thereof encoded by the pig H-FABP gene.

The invention further provides methods localising, identifying or marking genes or alleles or quantitative trait loci, in particular those corresponding to the pig H-FABP gene, in samples, in particular biological samples, cells or tissues, such as but not limited to hair, skin or blood, of farm animals, in particular pigs, by allowing for specific amplification of genomic fragments of those genes or alleles or quantitative trait loci of pigs. Since marker assisted selection of animals is frequentally based upon genetic variation that exists within functional genes that influence a production trait directly, i.e. genes such as the pig H-FABP that regulates fatty acid binding, one of the methods that the invention provides is a method that identifies or marks loci or genes and that can distinguish between characteristics of alleles of those genes which characteristics serve as markers in selection programmes for animals with specific versions of those genes that are directly linked with improved production traits.

The invention further provides a method wherein polymorphic restriction sites within functional genes and thus different alleles of those genes are identified by allowing for specific amplification of genomic fragments of those genes, in particular by allowing for specific amplification of fragments of the H-FABP gene. Amplification methods are well known in the art, the best known being PCR. A short description of the PCR used herein is given in the experimental part. Other primers, enzymes and conditions can of course be applied. After amplification a suitable method of identifying wanted alleles is a restriction endonuclease treatment. Suitable restriction enzymes for pig H-FABP alleles are *MspI*, *HaeIII* or *HinfI*, but others may also be used. By these methods large numbers of pigs can be rapidly genotyped for studies in which genotypic variation can be

associated with growth characteristics and other production traits of pigs.

However, there are many other methods identifying polymorphisms in alleles, both at the nucleic (DNA/RNA) level and at the product (protein) level. In particular at the protein level there are many possibilities using immunoassays, whereas at he nucleic acid levels there are many assays which all include some kind of hybridisation step of for instance primers or labelled nuclei acids. A very good possibility would be mismatch PCR. Primers to be used in the invention can be identified by the person skilled in the art, the sets given in the experimental part are for illustrative purposes only.

Furthermore, the methods according to the invention can be developed into diagnostic assays or kits by which selection of pigs with alleles of interest can be performed in routine screening protocols employed in breeding programmes. With such protocols better results of selection can be expected when genes responsible for regulation of commercially interesting body tissues can be rapidly identified and controlled.

In the specific case of the pig H-FABP gene, such testing protocols can be used to identify, select and breed farm animals, such as pigs, which have better production traits, such as IMF% or backfat thickness or average daily weight gain or feed efficiency, than the average animal in the population. Better production traits such as BW or daily weight gain will increase the production per year expressed as amount of meat per animal raised. A population of animals with a higher and less variable IMF% will result in a more homogenous product (meat) which is also better appreciated by putative customers because of a better taste. Furthermore, selection for higher IMF% may be possible while at the same time selection against fat deposition in other depots, such as backfat, can be performed.

EXPERIMENTAL

The porcine H-FABP gene has been isolated, characterized and chromosomally localized. Poylymorphisms in this gene have been identified. To test the association between bodyweight (BW) and percentage of intramuscular fat (IMF %), animals with different polymorphisms were selected, their bodyweight was measured and the amount of IMF after slaughter was measured.

MATERIALS AND METHODS

Isolation of H-FABP containing phage clones.

A porcine genomic DNA EMBL3/SP6/T7 library (Clontech Laboratories Inc. Palo Alto, CA) was screened using the plaque hybridization method (Sambrook et al. ,1989). The human H-FABP cDNA cloned in the pSP65 vector (Peeters et al.,1991) and labeled with 32P-dCTP by nick translation (Sambrook et al.,1989) was used as a probe . Briefly, 500.000 plaques were transferred to replica nitrocellulose filters and incubated in denaturation buffer (1.5 M NaCl, 0.5 M NaOH) for 2 minutes, neutralisation buffer (1.5 M NaCl, 0.5 M Tris-HCl pH 8.0) for 5 minutes and fixation buffer (0.2 M Tris-HCl pH 7.5, 2X SSC(0.3 M NaCl, 0.03 M Sodium citrate)) for 30 s. The filters were air dried and the DNA was irreversibly bound by baking the filters at 80°C for 2 h.

The filters were prehybridized (6X SSC, 0.5% (*w/v*) SDS, 5X Denhardt's and 100ug/ml NaOH treated salmon sperm DNA) for two hours at 67°C and hybridized in identical buffer with the addition of the radioactively probe at 67°C overnight. The filters were washed four times with 2X SSC, 0.1% (*w/v*) SDS for 30 min at room temperature. Twenty plaques that showed positive signals on both replica filters were isolated and each subjected to two additional rounds of low density plaque purification.

DNA of these clones was isolated using the plate lysate method (Sambrook et al.,1989).

Polymerase chain reactions.

PCR amplifications were performed on 1 µl of a 1:1000 dilution of phage DNA preparations or 50 ngr of genomic DNA in 50 µl containing 0.2 U Super Tth polymerase (SphaeroQ, Leiden, Nl) in 10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM MgCl, 0.1% (*w/v*) gelatin, 1% Triton X-100, 0.5 µM of each primer (Pharmacia Biotechnologies, Uppsala, Sweden) and 0.2 µM of each dNTP (Boehringer Mannheim, Mannheim, Germany). After 3 min of denaturation at 94°C, 33 cycles of amplification were carried out: 94°C for 1 min, the indicated annealing temperature (Tables 1 and 2) for 1 min and 72°C for the time considering the length of the expected fragment (approximately 1 min for every kb).

The primer sequences as used for the poly-T microsatellite amplification are

[Hex]-5'TCTGGGCTTCAACTTACTCTG3' and

5'CTAGCGCTTCAGCTCTGATTG3'

PCR was performed as described before with the following cycling conditions, 20 s at 94°C, 40 s at 57° and 1 s at 72°C followed by a final extension step for 10 min at 72°C, PCR products were analysed on a 8% polyacryl amide gel in an ABI373A (Perkin Elmer, Foster City, CA, USA) using Genescan 1.0/Genotyper 1.1.1.2 software (Perkin Elmer). Allele sizes were estimated by comparison with a commercial Tamra-labeled marker (Perkin Elmer).

DNA sequence analysis

PCR#1 (Table 1) was performed on DNA of all the phage clones to detect porcine H-FABP intron 1 specific fragments. Two of the three phage clones containing the H-FABP gene were used to subclone the various *Sac*I and *Kpn*I (Boehringer

Mannheim, Mannheim, Germany) restriction digestion fragments of the gene region in pBS. Unfortunately, neither of these phage clones contained exon 4 and the 3' untranslated region as detected by restriction analysis.

The intron 3/exon 4 splice junction was cloned as PCR#2 (Table 1) product using porcine (Great Yorkshire) genomic DNA as a template. The products of two independent PCR reactions were cloned to identify errors by the Super Tth polymerase upon analysis.

The 3' untranslated region was isolated using the 5'/3' RACE-PCR kit (Boehringer Mannheim, Mannheim, Germany) with porcine (Meishan) muscle cDNA as the template and porcine H-FABP exon 1 or 3 (Table 1) specific primers in combination with the provided poly-A primer.

PCR products were cloned in the pT7Blue vector (Novagen Inc.,Madison, U.S.A.).

All H-FABP (sub)clones were transformed and the recombinant plasmid DNA was isolated and purified with the Wizard Maxiprep kit (Promega, Madison, WI, U.S.A.). The nucleotide sequence was determined by dideoxy sequencing, partially by cycle sequencing (Perkin Elmer,) or autoread sequencing (Pharmacia Biotechnologies, Uppsala, Sweden) and the analysis was performed on a ABI 373 (Applied Biosystems) or ALF DNA sequenator (Pharmacia Biotechnologies, Uppsala, Sweden) respectively.

RFLP screening

Porcine genomic DNA was isolated as described (Sambrook et al.,1989) from EDTA treated blood stored at -80°C. One hundred ng of genomic DNA was used for PCR amplification in 50 µl reaction as described before. The primer sequences and its corresponding product size and annealing temperature for each combination are given in table 2. Fifteen µl of the PCR reaction was used for restriction digestion with 2 units of *Hae*III, *Hin*fI or *Msp*I (Boehringer Mannheim) in a total volume of 20 ul. For *Hae*III and *Hin*fI the recommended buffer

conditions were additionally used whereas *Msp*I was added directly to the PCR buffer. Restriction digestion fragments were loaded on a 2% (*Msp*I) or 3% (*Hae*III and *Hin*fI) agarose (Sigma, St Louis, MO, U.S.A.) gel and after electrophoresis the RFLP patterns were scored by two persons, independently.

Chromosomal localisation

Two independently established pig/rodent somatic cell hybrid panels (Panel A: Rettenberger et al.,1994a,1994b,1994c, 1995a and Panel B: Zijlstra et al.,1994a,1994b,1994c, in prep.) were used to assign the H-FABP gene to a specific chromosome by PCR.

DNA from each cell hybrid containing porcine chromosomes in various combinations was used in PCR#3 (Table 1) which unambiguously amplified porcine H-FABP intron 3 sequences. The obtained data was statistically evaluated according to Chevalet and Corpet (1986) in comparison with the cytogenetically and/or reference loci data of both panels.

Analysis of bodyweight and intramuscular fat in relation to polymorphisms in H-FABP genotypes.

One hundred Duroc pigs were selected on the basis of the amount of IMF in their slaughtered relatives. The animals came from the test farms Someren (N=50) and Herpen (N=50). Blood samples were used to isolate DNA which was used to determine the genotypes of the three polymorphisms of each animal. Of all animals BW was measured. Animals which where not used in the regular breeding program where slaughtered and analyzed for the amount of IMF. Only 45 animals were slaughtered. For three of these animals it was not possible to determine the genotype of one or more polymorphisms. In total 42 animals were used in the analyses.

An additional experiment was performed to further study the production traits in relation to genetic variation in the H-FABP gene. Therefore thirteen boars and seventy-two dams were selected for this investigation from two Duroc populations housed at separate test stations. Selection was based on heterozygosity for each H-FABP PCR-RFLP. Progeny was housed in groups and fattened with ad libitum food access until slaughterweight (110 kg).
Performance traits recorded for each pig were live weight at 180 days of age (BW), backfat thickness (BFT) and for each dam the number of piglets produced alive in first (FPP) or second parity (SPP), respectively. At slaughter, meat quality traits i.e. cooking loss, drip loss, intamuscular fat percentage, minolta colour, pH and shear force were measured in a subset of the slaughtered animals.
Blood or hairroots were collected from each animal to isolate genomic DNA for H-FABP PCR-RFLP genotyping.
The final dataset comprises information from in total 2345 pigs including pedigree. For 823 pigs H-PABP genotype information is available for at least one of the PCR-RFLPS.

Analysis was done with the statistical program Statistical Analyses System (SAS, 1990) and with the program Prediction and Estimation (PEST, Groeneveld, 1990). The latter program uses family information to estimate Best Linear Unbiased Predictions (BLUP) for the influence of, in this case different genotypes on the amount of IMF and BW. The mean amount of IMF% of the Duroc population is 3.20, its standard deviation is 0.84 (Hovenier, 1992). PEST was also used in combination with the GeneProb program (Kerr and Kinghorn, 1996) that estimates missing genotypes for animals based on genetical and or phenotypical information for the trait of interest. Thus, the effect of the H-FABP genotypes on performance, and meat quality traits was studied with the following model:

Trait= int + test station*test year*test month + sexe + litter + P(XX) + P(Xx) + P(xx) + individual + covariable + residual effect

where
P(XX), (Xx), (xx) are the estimated chance for each genotype for each animal.
BW was standarized to weight at 180 days (STD-BW)
BFT was standarized to a weight of 110 kg (STD-BFT)
For IMF age was included as a covariable and analysis was also performed with STD-BFT and STD-BW as covariables.
Heritability (h^2) estimates for each trait in this Duroc population were assumed to be similar to the estimates described by Hovenier et al. (1992) for the Duroc breed.

RESULTS

H-FABP gene sequence determination and analysis

Twenty H-FABP positive phage clones were identified and the corresponding DNA isolated and examined for the presence of the H-FABP gene. Using PCR#1 (Table 1) three phage clones appeared to contain intron 1. The rest of the phage clones contained H-FABP pseudogene-like sequences because of the absence of intron 1 and 3 in the amplification product of PCR#1 and #2 (Table 1) respectively. Sequence analysis of these pseudogene specific amplification products showed various nucleotide substitutions in comparison with the H-FABP gene coding sequences. Furthermore a 27 bp internal duplication was detected in the PCR#3 amplification product of one H-FABP pseudogene containing phage clone (data not shown). However this particular pseudogene specific PCR#3 fragment was not detected in the main pig breeds of our panel.

Further PCR analysis of the three H-FABP gene containing phage clones revealed that neither contained the exon 4 and 3' untranslated region of the gene. Using PCR#2

on porcine genomic DNA a 1500 bp fragment was amplified and cloned for intron 3 and exon 4 sequence analysis. The 3' untranslated region (3'UTR) was amplified on porcine whole muscle cDNA, cloned and sequenced.

The coding sequences with the flanking intronic sequences and also 1600 bp of the 5' upstream region were determined (Fig.1). The exon-intron splice junctions were located in comparison with the porcine H-FABP cDNA and the murine H-FABP/MDGI (Treuner et al.,1994) gene sequence. A potential TATA-box was located 92 bp upstream the ATG start codon and in the (3'UTR) a consensus poly-A signal sequence was identified (see Fig.1). The coding sequences showed 92%, 91%, 87% and 85% identity to the bovine, human, mouse and rat H-FABP sequences at the nucleotide level and the deduced amino acid sequence were 92%, 90%, 87% and 86% identical, respectively (Billich et al., 1988; Peeters et al., 1991; Binas et al., 1992; Claffey et al., 1987).

Detection of genetic variation

A panel comprising genomic DNA of 7 pig breeds each represented by unrelated animals (see table 3) was used to detect genetic variation in the 5' upstream region, intron 2 and intron 3 of the porcine H-FABP gene. Therefore, part of the 5' upstream region was amplified on DNA of this panel using PCR (Table 2) and digested with the restriction enzyme *Hinf*I. The *Hinf*I digestion showed two alleles a single fragment of 256 bp (allele h) or two fragments of 197 and 59 bp (allele H). Similarly intron 3 (PCR#3,Table 1) and intron 2 (Table 2) were tested for genetic variation with the enzymes *Msp*I and *Hae*III respectively, and both showed genetic variation in intron 2. *Hae*III showed one fragment of 850 bp (allele D) and/or fragments of 400 and 450 bp (allele d). Accurate size determination revealed that these three fragments were 684 bp, 278 bp and 406 bp. *Msp*I showed a fragment of 850 bp (allele a) and/or fragments of 750 and 50 bp (A). Accurate size determination revealed that these

fragments were 814 bp, 703 bp and 111 bp. Both sites of genetic variation are approximately 300 (285) bp apart.

Sequence analysis of the porcine H-FABP gene sequence (Fig. 1) revealed a 25 thymidine-nucleotide (poly-T microsatellite) stretch in the first intron. To investigate genetic variation in this poly-T stretch this region was amplified by PCR. In the Duroc pigs at least 3 alleles (H1, 215-bp, H2:220-bp and H3:221-bp) were detected. Obviously, these alleles showed a complete linkage with H-FABP PCR-RFLP alleles which are located within a 1,5 kb region.

Table 3 represents the allele frequencies of the different PCR-RFLPs in the different pig breeds tested.

The mendelian inheritance pattern of the three PCR-RFLPs was analysed in a porcine family comprising 3 generations of a Great Yorkshire breed. The genotypes of the individual pigs show consistent patterns of inheritance in this family.

Chromosomal localisation

The porcine H-FABP gene was chromosomally localized using a porcine H-FABP gene intron 3 specific PCR which amplified no rodent homologous. Amplification on DNA of two independently established pig/rodent cell hybrid panels and comparison with the cytogenetically (panel A and B) and reference loci data (panel A) revealed a single significant association of the H-FABP gene with chromosome 6 (Table 4) for both cell hybrid panels.

Analyses of bodyweight and intramuscular fat in relation to polymorphisms in H-FABP genotypes.

Table 5 shows the result of mean values and their standard deviations of IMF and bodyweight for different fixed effects which were taken into account in this analyses.

Statistical analyses using SAS

The data was analyzed using the General Linear Models procedure (GLM) from SAS (Sas, 1990). A large model (lrg) with IMF as dependant variable contained the (fixed) effects test farm, sexe, *Msp*I, *Hae*III and *Hin*fI. Also models containing the different effects separately (ind) and one containing the combined genotype of these three polymorphisms were analyzed. Table 6 shows the significance values for the different effects for the models analyzed. Table 9 shows the effects of the three polymorphisms on intramuscular fat%.

Statistical analyses using PEST

The PEST program was used to be able to use family information in the analyses of the different fixed effects. The used model contained the same fixed effect as the model with SAS but also contains a random animal effect. Also, a pedigree file was used containing family relations up to two generations back. Table 7, 10, 11, 12 and 13 show predicted values for the different fixed effects and their standard errors.

Hypothesis testing

The different values and their standard errors, found for the fixed effects where used in a Chi-squared hypothesis test. Critical values were calculated for a 90% and 95% two-sided confidence limit. Calculation was performed using formula 1 and 2. The $\mu 1$, $\mu 2$, s.e.1 and s.e.2 are taken from PEST output.

The value $t1\text{-}1/2\tau$ is taken from a confidence table and has a value of 1.96 for a 95% confidence interval.

$$y1\text{-}y2 = \mu 1 - \mu 2 \pm t1\text{-}1/2\tau * \sqrt{(s.e.2)}$$

$$s.e.2 = (n1 * s.e.12 + n2 * s.e.22)/(n1+n2-2)$$

$t1-1/2\tau$ = 1.96 (95% confidence limits)

$\mu1-\mu2$ = difference between two genotypes of one polymorphism

Table 8 shows the difference between different genotypes of each polymorphism and its 90% and 95% critical values.

The differences between the values for the different genotypes on IMF are not significant (90%). Only the difference between the homozygote combination (aaddHH) and the heterozygote combination (AaDdHh) showed a significant difference (90%) but was not significant with the 95% two sided confidence limits.

Bodyweight is significantly different for the different genotypes in all three polymorphisms.

When the different genotype combinations are analysed for bodyweight the heterozygote combination (AaDdHh) did not differ significantly from both homozygote genotypes. Both homozygote genotypes (AADDhh-aaddHH) show a significant difference of 9.11 kg ($P<0.05$).

Conclusions

The genotypes of the three polymorphisms tested (*MspI*, *HaeIII* and *HinfI*) show a significant (95%) difference in bodyweight (BW). All three polymorphisms can be used in selection for bodyweight. The genotypes of the three polymorphisms show a distinct, albeit non-significant difference in IMF percentage. If there is a difference between different genotypes of 0.20, 50 animals of the least frequent genotype (AADDhh) would be needed to reach a significant (95%) difference of 0.2. Tables 9, 10 and 11 show that when more animals are tested, statistically significant differences among the three polymorphisms can indeed be found, for instance for IMF, backfat thickness and BW.

Also, tables 12 and 13 show that the effect on IMF, as measured by RFLP testing, can stil be found when the effects are corrected for backfat thickness and/or growth.

Brief description of the drawings

Figure 1. The porcine H-FABP gene sequence including 1632 bp of the 5'upstream region and 200 bp of the 3' untranslated region. Exons are represented by bold capital letters and the deduced amino acid sequence is shown directly beneath it. Standard one letter amino acid symbols are used. The putative TATA-box, the polyadenylation signal in the 3'UTR and the 13 nucleotide element are underlined. The size of the nondepicted intron sequences is shown between arrowheads. The polymorphic *Hae*III (GGCC), *Hinf*I (GATTC), *Msp*I (CAGG) sites and the polymorphic microsatellite sequence (poly-T) are depicted bold and underlined.

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Table 1: Primer sequences and the corresponding size and annealing temperatures for PCR reactions of different regions of the porcine H-FABP gene.

PCR	position	primer sequence	T(°C)	size (bp)
PCR#1	exon 1	5'GCCAGCATCACTATGGTGGACGCTTTC	57	4000
	exon 2	5'CTTAAAGCTGATCTCTGTGTTC		
PCR#2	exon 2	5'GGAGGCAAACTTGTTCACCTGC	57	1600
	exon 4	5'TCTTTCTCGTAAGTGCGAGTGC		
PCR#3	exon 3	5'GGAGGCAAACTTGTTCACCTGC	62	1500
	intron 3	5'GTACTGGGAGCACTCTTCACTC		

Table 2: The primer sequences and combinations used for PCR-RFLP detection with the corresponding annealing temperature.

RFLP	Primer sequence	T (°C)	size (bp)
HaeII-I/MspI	5'ATTGCTTCGGTGTGTTTGAG	57	950
	5'TCAGGAATGGGAGTTATTGG		
HinfI	5'GGACCCAAGATGCCTACGCCG	57	700
	5'CTGCATCTTTGACCAAGAGG		

Table 3: Allele frequencies of the porcine H-FABP gene *HaeIII*, *MspI* and the *HinfI* RFLPs in unrelated animals of different pig breeds.

		DL	Du	GY	Hs	Me	Pi	WP
		20	10	34	6	11	5	5
MspI	AA	0.95	0.20	0.65	1.00	1.00	0.80	0.40
	Aa	0.05	0.40	0.32	0.00	0.00	0.20	0.60
	aa	0.00	0.40	0.03	0.00	0.00	0.20	0.00
HaeIII	DD	0.05	0.20	0.06	1.00	1.00	0.40	0.00
	Dd	0.55	0.40	0.50	0.00	0.00	0.20	0.20
	dd	0.40	0.40	0.44	0.00	0.00	0.40	0.80
HinfI	HH	0.45	0.60	0.94	0.17	0.18	0.60	0.80
	Hh	0.50	0.20	0.06	0.33	0.55	0.20	0.20
	hh	0.05	0.20	0.00	0.50	0.27	0.20	0.00

Abreviations for the various pigbreeds are DL: dutch Landrace; DU: Duroc; GY: Great Yorkshire; HS: Hampshire; ME: Meishan; PI: Pietrain; WP: Wild Pig. The second line indicates the number of unrelated animals tested.

Table 4: Chromosomal localization of the porcine hFABP gene by two independent cell hybrid panels.

The percentage concordance was determined by the total of equal absence/presence in both data sets divided by 20. The φ value represents the correlation between both datasets. A gene can be assigned to a chromosome when the concordance is high and the φ is more than 0.74 (syntenic). Values for φ between 0.59 and 0.74 give no validation of the assignment. The rest is asyntenic. Using these conditions the probability of the assignment is 97.5% correct.

		PANEL A		PANEL B	
CHR.NR	locus	%CON	φ	%CON	φ
1	IFNA	62	0.16	46	-0.10
2	S0091	81	0.59	71	0.39
3	APOB	67	0.39	66	0.45
4	S0001	48	-0.03	66	0.34
5	S0092	71	0.45	50	0.06
6	RYR1	**95**	**0.89**	**92**	**0.83**
7	TNFB	62	0.33	62	0.10
8	ALB	48	0.31	62	0.36
9	S0095	57	0.40	50	0.15
10	S0038	57	0.28	50	0.03
11	CGT9	48	-0.03	58	0.17
12	GH	57	0.40	50	-0.03
13	S0076	48	-0.03	67	0.29
14	DAO	62	0.16	71	0.45
15	S0088	52	0.03	75	0.53
16	CGT6	57	0.28	58	0.10
17	ENDO	67	0.30	50	-0.15
18	CGT12	57	0.00	58	0.08
X	S0022	52	0.12	75	0.54
Y	SRY	76	0.39	58	0.00

Panel A: Rettenberger et al., 1994a
Panel B: Zijlstra et al., 1994a

Table 5 Number of animals (N), their mean and standard deviations (std) for intramuscular fat (IMF) and bodyweight (BW) difference from 110 kg at 180 days) for different fixed effects (effect).

Effect	N	IMF(%)	(std)	BW (kg)	(std)
test farm					
Someren	24	2.77	(1.66)	-4.87	(8.35)
Herpen	18	2.00	(0.79)	-6.73	(7.85)
Slaughter week					
1	5	2.02	(0.54)	-4.76	(8.34)
2	19	2.25	(0.77)	-5.83	(6.81)
4	6	2.82	(0.70)	-6.12	(8.80)
5	5	2.20	(1.11)	-8.65	(11.61)
6	7	3.09	(3.02)	-3.34	(9.63)
Slaughter month					
jan	35	2.31	(0.79)	-6.13	(7.83)
feb	7	3.09	(3.02)	-3.34	(9.63)
Sexe					
boar	24	2.02	(0.71)	-3.44	(7.90)
gilt	18	2.99	(1.85)	-8.63	(7.57)
Polymorphisms					
*Msp*I					
AA	5	2.58	(0.69)	-10.27	(6.78)
Aa	24	2.54	(1.73)	-7.23	(7.47)
aa	13	2.18	(0.80)	-1.79	(8.01)
*Hae*III					
DD	5	2.58	(0.69)	-10.27	(6.78)
Dd	22	2.57	(1.80)	-7.26	(7.53)
dd	15	2.19	(0.76	-1.01	(8.12)
*Hin*fI					
HH	21	2.02	(0.72)	-4.04	(9.06)
Hh	15	3.00	(2.04)	-6.50	(7.00)
hh	5	2.58	(0.69)	-10.27	(6.78)

Table 6 Values of significance for different fixed effects influencing intramuscular fat (IMF) and bodyweight (BW) using a large model (lrg) and using seperate models per fixed effect (ind) (correcting for fixed effects test farm and sexe).

Effect	IMF (Pr > F)		BW (Pr >F)	
	lrg	ind	lrg	ind
regio	0.07	-	0.99	-
sexe	0.02	-	0.03	-
*Msp*I	0.52	0.38	0.39	0.03
*Hae*III	0.60	0.50	0.56	0.04
*Hinf*I	0.55	0.43	0.59	0.30
Combined	-	0.80	-	0.11

Table 7 Predicted values for intramuscular fat (IMF) and bodyweight (BW) for the different fixed effects (effect) and their standard errors (s.e.).

Effect	IMF s.e.	BW s.e.
sexe		
boar	-0.61±0.18	4.78±2.52
test farm		
Someren	0.48±0.24	2.56±3.05
*Msp*I*		
AA	-0.02±0.32	-3.78±4.39
Aa	0.00±0.00	0.00±0.00
aa	-0.22±0.23	5.34±3.07
*Hae*III*		
DD	0.02±0.32	-4.32±4.36
Dd	0.00±0.00	0.00±0.00
dd	-0.12±0.22	4.35±2.87
*Hinf*I*		
HH	-0.30±0.21	2.16±2.84
Hh	0.00±0.00	0.00±0.00
hh	-0.08±0.32	-5.16±4.36

* the different polymorphisms are analysed using one polymorphism in each model (the fixed effects test farm and sexe are included in all models).

Table 8 Differences (diff) between genotypes and their 90% (90) and 95% (95) confidence limits for intramuscular fat (IMF) and bodyweight (BW).

genotype	IMF diff	IMF 90	BW diff	BW 95
aa-AA	-0.20	±0.39	9.11	±8.23
dd-DD	-0.14	±0.39	8.56	±6.48
HH-hh	-0.22	±0.38	7.32	±6.43

Table 9 Effect of different genotypes and the difference between the homozygotes in IMF% for each RFLP

Genotype	effect	difference	P=0
MspI			
AA	-0.373		
Aa	-0.250	-0.373 ± 0.195	0.06
aa	0.000		
HaeIII			
DD	-0.379		
Dd	-0.274	-0.379 ± 0.191	0.05
dd	0.000		
HinfI			
HH	0.402		
Hh	0.048	0.402 ± 0.191	0.04
hh	0.000		

Table 10 Effect of different genotypes on backfat thickness (difference from population mean at 110 kg in mm)

Genotype	effect	difference	P=0
MspI			
AA	-0.559		
Aa	-0.346	-0.559 ± 0.209	0.01
aa	0.000		
*Hae*III			
DD	-0.598		
Dd	-0.342	-0.598 ± 0.207	0.004
dd	-0.000		
*Hinf*I			
HH	0.380		
Hh	0.092	0.380 ± 0.213	0.07
hh	0.000		

Table 11 Effect of different genotypes on bodyweight (difference from population mean at 180 days in kg)

Genotype	effect	difference	P=0
*Msp*I			
AA	-2.311		
Aa	-2.229	-2.311 ± 1.275	0.07
aa	0.000		
*Hae*III			
DD	-2.691		
Dd	-2.300	-2.691 ± 1.258	0.03
dd	0.000		
*Hinf*I			
HH	0.404		
Hh	-0.621	0.404 ± 1.298	0.76
hh	0.000		

Table 12. Effect of different genotypes and the difference between the homozygotes in IMF% for each RFLP when corrected for backfat thickness.

Genotype	Effect	Difference
*Msp*I		
AA	-0.226	-0.226 ± 0.188
Aa	-0.166	
aa	0.000	
*Hae*III		
DD	-0.234	-0.234 ± 0.184
Dd	-0.195	
dd	0.000	
*Hinf*I		
HH	0.316	0.316 ± 0.182
Hh	0.006	
hh	0.000	

Table 13 Effect of different genotypes and the difference between the homozygotes in IMF% for each RFLP when corrected for backfat thickness and growth

Genotype	Effect	Difference
*Msp*I		
AA	-0.245	-0.245 ± 0.188
Aa	-0.192	
aa	0.000	
*Hae*III		
DD	-0.250	-0.250 ± 0.184
Dd	-0.214	
dd	0.000	
*Hinf*I		
HH	0.316	0.316 ± 0.182
Hh	0.001	
hh	0.000	

CLAIMS

1. An isolated or recombinant pig H-FABP gene specific nucleic acid molecule or pig H-FABP gene specific fragments thereof comprising or hybridising to the nucleotide sequence as listed in figure 1 or its complementary sequence or the RNA equivalents thereof .

2. A method localising, identifying or marking genes or alleles or quantitative trait loci of pigs, using a molecule or a fragment or fragments thereof according to claim 1.

3. A method according to claim 2 whereby genes or alleles or quantitative trait loci are localised, identified or marked that are associated with production traits of pigs.

4. A method according to claim 2, or 3 to identify or mark alleles of the pig H-FABP gene.

5. A method according to claim 4 distinguishing between alleles of the H-FABP gene of pigs.

6. A method according to claim 5 by detecting specific restriction sites in an allele of the H-FABP gene of pigs.

7. A method according to claim 6 whereby an *MspI* restriction site is detected.

8. A method according to claim 6 whereby an *HaeIII* restriction site is detected.

9. A method according to claim 6 whereby an *HinfI* restriction site is detected.

10. A method using a molecule, a fragment or fragments thereof according to claim 1, localising, identifying or marking genes or alleles or quantitative trait loci in samples, in particular biological samples, cells or tissues, such as but not limited to hair, skin or blood, by allowing for specific amplification of genomic fragments of those genes or alleles or quantitative trait loci.

11. A diagnostic assay or kit according to claim 10.

12. A method according to anyone of claims 2-9 localising, identifying or marking genes or alleles or quantitative trait loci in samples, in particular biological samples,

cells or tissues, such as but not limited to hair, skin or blood of pigs, by allowing for specific amplification of genomic fragments of those genes or alleles or quantitative trait loci.

13. A method according to anyone of claims 10 or 12 in which the method of amplification is the polymerase chain reaction.

14. Diagnostic assay or kit according to claim 13.

15. A method according to anyone of claims 2-14 identifying differences between alleles of the pig that are associated with differences in production traits of pigs.

16. A method according to claim 15 identifying alleles of the pig that are associated with improved production traits of the pig.

17. Use of the methods according to anyone of claims 2-16 in marker assisted identification of pigs or in marker assisted selection of pigs.

18. Use of the methods according to claims 16 or 17 in breeding programmes.

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Figure 1/1

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S I V

CACTGGATGGAGGCAAACTTGTCCACCTGCAGAAGTGGAATGGACAAGAGACAACGCTTGTTC
T L D G G K L V H L Q K W N G Q E T T L V

GGGAACTAGTTGATGGGAAACTCATCCTGgtaagatggacaactttggagtctcacccagttg
R E L V D G K L I L

gttcttactgcccctcagccaaccctactgtgaaaagccaaggttccctgggaccagtggaa
gctgggatagtggagggtggaggcaaggctgggtggcattaggcattaggtaggaattttctt
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agagtgctcccagtaccttctgtgcccttacccctgccctaatctgaccctcttttattccag
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T L T H G S A V C T R T Y E K E A stop

cccttcgactgttcctgccaattggctactcctggactcagcaccagattgcctcatttttc
ctctggcattttgtaaaaatctactttggggatattctcctggggtcaggttgcaccagcctg
cgttcagttccggttcttgttgtgtatgttagttttttttaattgcatccaaagggtgctct
gaggtc<u>aataaa</u>atagccaaggc

Figure 1/2

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(57) Abstract

The present invention provides a novel sequence of the pig H-FABP gene, as well as methods of using said gene and its products. Especially the invention provides methods for detecting different alleles of the pig H-FABP gene, which different alleles are associated with differences in the genotypic and/or phenotypic traits of the pigs having those alleles. Especially the invention provides methods for distinguishing between alleles resulting in different phenotypes, particularly using techniques involving selective amplification of pig H-FABP gene derived materials. These techniques are especially suitable for selecting animals to be used in breeding programmes. Breeding programmes employing such techniques are also disclosed.

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INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
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B. FIELDS SEARCHED

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

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[X] Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA	Authorized officer
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Inter nal Application No
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